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## **Threshold Effects of Food Concentration on the Skeletal Morphology of the Bryozoan *Electra Pilosa* (Linnaeus, 1767).**

**Steven J. Hageman, Lyndsey L. Needham and Christopher D. Todd**

### **ABSTRACT**

Many palaeontological studies rely heavily on characteristics of the preserved phenotype, i.e. the morphology of skeletal hard parts. Although the potential for environmental influences on the phenotype is expected, rarely is the magnitude of the effects quantifiable relative to genetic factors. The clonal/colonial body plan of Bryozoa allows for the partitioning of morphological variance into its genetic and environmental factors addressing the question of, 'how much phenotypic variation is induced in a population by changing a single environmental factor?' The effects of variation of food concentration on whole-colony growth rate and on zooid size/morphology can be profound in bryozoans. Here we test experimentally food effects on the skeletal phenotype of the bryozoan *Electra pilosa* (Linnaeus, 1767), an encrusting sheet-like bryozoan. A threshold effect was observed for the relationship between zooecium size and food concentration. Very low concentrations resulted in stunted colonies with small zooecia, but at low to intermediate concentrations a close relationship existed with zooecium size. Maximum zooecium size occurred at submaximal food concentration and submaximum zooecium size occurred at higher food concentrations. Previous studies that have reported no effect of food availability on zooecium size assessed food concentration effects at higher concentrations than were effective in the present study. In the absence of other factors, variation in zooecium size is minimal and unchanging at moderate to high food concentrations. Greater variation in zooecium size is expected at and below threshold food concentrations. We show that the preservable phenotype of these specimens subjected to controlled and induced environmental variation also records information with genetic significance.

## ARTICLE

The aim of this study is to address the question: 'what effects do varied food concentrations have on the hard-part morphology of the marine bryozoan *Electra pilosa* (Linnaeus 1767)?' Understanding factors that control the phenotype, especially preservable hard parts, is fundamental to the identification and recognition of fossil species and to any study of microevolutionary patterns and processes. Phenotypic variation embraces all the visible differences among organisms of the same biological species. The phenotype of a given individual is controlled by its genetics, environmental conditions and their interactions (Falconer & MacKay 1996). In order to evaluate the reliability of the phenotype as a proxy for genetic variation/isolation of fossil species, an understanding of the relative contribution of genotype, environment and genotype by environment interaction is essential (Hageman et al. 1999, 2002).

Many studies have documented phenotypic variation within species using a wide range of both fossil and recent material (e.g. Oliver 1960; Koepnick & Kaesler 1971; Makurath & Anderson 1973; Malmgren & Kennett 1976; Best et al. 1984; Reyment et al. 1988; Meyer & Ausich 1997; Wang et al. 2004). Previous work also demonstrated differences among phenotypes from different environments (e.g. Farmer & Rowell 1973; Kahn 1981; Werdelin & Hermelin 1983; Baumfalk et al. 1987; Herrera & Jackson 1992). Few studies have, however, partitioned variation into its genetic and specific environmental sources (e.g. Cheetham et al. 1993, 1995; Hunter & Hughes 1994; Bayer et al. 1997; Riisgård & Goldson 1997; Hageman et al. 1999, 2002).

Bryozoans are especially tractable experimental animals for morphometric studies because they are clonal with individual zooids (modules) budded asexually within the colony (Farmer & Rowell 1973; Hageman 1995; Holdener & Hageman 1998). These characteristics allow for individuals of distinct genotypes to be cloned by fragmentation and grown simultaneously under different but controlled environmental conditions (Jebram 1973, 1975; Hunter & Hughes 1994; Bayer et al. 1994; Bayer et al. 1997; Riisgård & Goldson 1997; Hermansen et al. 2001).

Here we address the effects of a controlled environmental factor on phenotypic variation by manipulating a single, but ecologically relevant, environmental factor (food concentration) for genetically identical (clonal) replicates of the marine bryozoan *Electra pilosa*. In addition, we show that the skeletal phenotype of these, specimens subjected to controlled and induced environmental variation, also records information with genetic significance.

### Effect of food concentration in Bryozoa

Previous studies have yielded contradictory conclusions concerning the effect of food concentration on hard part zooecial morphology in bryozoans. Estimates of individual zooecium sizes have previously been used to evaluate the importance of food on the skeletal phenotype of bryozoans (Jebram & Rummert 1978; Okamura 1987, 1992; Bayer et al. 1994; Hunter & Hughes 1994; Riisgård & Goldson 1997; O'Dea & Okamura 1999, 2000a,b; Hermansen et al. 2001). Positive correlation was reported for zooecium size versus food quality in *Conopeum seurati* (Jebram 1973, 1975) and versus food quantity in *Electra pilosa* (Riisgård & Goldson 1997; Hermansen et al. 2001). Bayer et al. (1994) showed that food concentration affected

overall colony growth rate in *Electra pilosa*. In contrast, no food effect on the number of zooids per unit area was reported either for *Electra pilosa* and *Conopeum reticulum* by Menon (1972) or for *Celleporella hyalina* by Hunter & Hughes (1994) grown in the laboratory under varied food and temperature conditions. Dudley (1973), Jebram (1980), Silén (1987) and Okamura (1992) all showed food to have an effect on colony form of bryozoans. Okamura (1987), Okamura & Bishop (1988), O'Dea and Okamura (1999, 2000a), O'Dea (2005), O'Dea et al. (2007), Lombardi & Cocito (2006), and Amui-Vedel et al. (2007) reported zooecium morphology to be unaffected by food concentration but showed a linear relationship with temperature. Schäfer (1994) and Berning (2007) attributed rhythmic variations of branch thickness to seasonal nutrition cycles in settings where annual thermal gradients are minimal, whereas Lombardi et al. (2008) demonstrated a correlation between variations in frond thickness and temperature.

## Materials and methods

Two data sets were used in this study. The first, based on the frontal surface area of individual zooecia of *Electra pilosa*, was used to compare zooecium size among colonies grown under controlled food concentrations and to compare those conditions to typical nutrition levels found in the natural environment. The second dataset permitted an evaluation of change in five morphometric characters under controlled conditions of varied nutrition levels for specimens grown in the laboratory. Data from these data sets were analysed separately.

### Data Set One: zooecium area

Data Set One consists of estimates of the frontal area of zooecia within and among colonies compiled from three sources (Table 1). Colony area divided by number of complete zooids was used as a proxy for zooecium area from tables in Riisgård & Goldson (1997) and Hermansen et al. (2001). Zooecium areas were measured from the same specimens of colonies reported by Bayer et al. (1994) (see Data Set Two for laboratory methods). Data are not directly comparable among studies (different laboratory conditions, protocols and methods), although trends within data sets can be compared with confidence.

**Table 1.** Summary of Data Set One. Average values for zooecium frontal areas (ZA) from three data sources (used in Fig. 3). Multiple genotypes (replicate clonal colonies) were grown under each Food concentration within each independent study. CV = coefficient of variation (standard deviation/average \* 100) in percentage. Number of observations for Bayer *et al.* (1994) = 10 per Genotype × Food concentration. Values for Riisgård & Goldson (1997) and Hermansen *et al.* (2001) were derived by dividing number of zooids by total area of zooecia grown by each genotype under each Food concentration.

Bayer *et al.* (1994)

<i>Rhodomonas</i> sp. cells/mL	Genotype			Average	CV
	1	2	3		
100	0.124	0.103	0.125	0.117	10.5
1000	0.147	0.108	0.157	0.137	18.8
10 000	0.188	0.153	0.173	0.171	10.2
100 000	0.171	0.156	0.147	0.158	7.6

Riisgård & Goldson (1997)

<i>Rhodomonas</i> sp. cells/mL	Genotype				Average	CV
	1	2	3	4		
810	0.301	0.258	0.312	0.312	0.296	8.6
2460	0.366	0.386	0.358	0.287	0.349	12.5
4620	0.429	0.443	0.403	0.504	0.445	9.6
8380	0.270	0.297	0.317	0.278	0.290	7.1
18 500	0.330	0.329	0.306	0.317	0.320	3.6

Hermansen *et al.* (2001)

<i>Rhodomonas</i> sp. cells/mL	Genotype					Average	CV
	1	2	3	4	5		
1000	0.112	0.145	0.190	0.178	0.163	0.158	19.4
1500	0.178	0.207	0.225	0.264	0.248	0.224	15.0
2500	0.183	0.189	0.179	0.197	0.231	0.196	10.6
3300	0.244	0.161	0.338	0.171	0.292	0.241	31.7
4100	0.250	0.218	0.225	0.258	0.220	0.234	7.8
5000	0.151	0.144	0.161	0.163	0.140	0.152	6.6

Clausen and Riisgård (1996) demonstrated the following relationship between the number of cells (C) of *Rhodomonas* ( $C, \times 10^3$  cells/mL) and concentration of chlorophyll *a* ( $\mu\text{g chl } a/\text{L}$ ):  $a = 1.251 C$ . That relationship was used to compare laboratory conditions under which the present *Electra pilosa* specimens were grown to nutrition levels in the natural environment and typical of waters from which specimens were originally collected (Table 2).

Table 2. Comparison of chlorophyll *a* concentrations in the natural environment with food concentration equivalents of the cryptophyte *Rhodomonas* sp. used in experimental laboratory conditions. For field locations (shallow nearshore), the observed values of chlorophyll *a* in bold are converted to the equivalent number of cells of *Rhodomonas* sp. in italics using equation in text from Clausen & Riisgård (1996). For laboratory experiments, the measured values for the number of cells/mL of *Rhodomonas* sp. are in bold, and the equivalent chlorophyll *a* µg/L values are in italics.

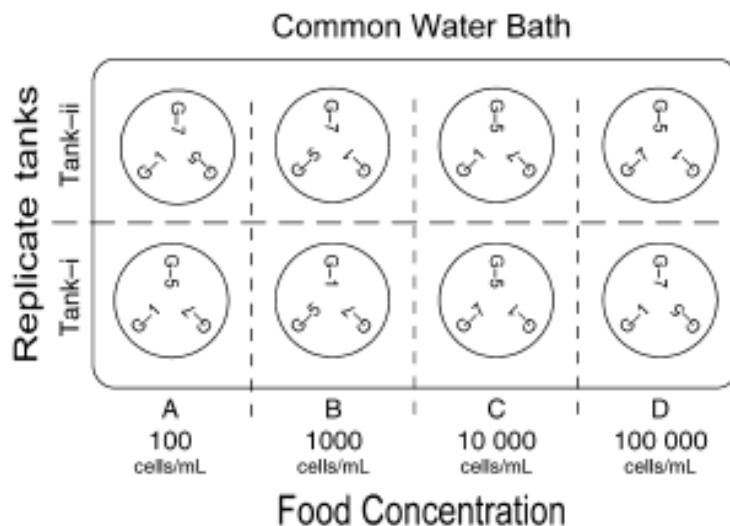
Location	Study	<i>Rhodomonas</i> sp. cells/mL	Chlorophyll <i>a</i> µg/L
Church Island	Hermansen <i>et al.</i> (2001) minimum	205	<b>0.256</b>
Menai Strait	Blight <i>et al.</i> (1995) minimum	400	<b>0.5</b>
Malin Shelf	Gowen <i>et al.</i> (1998) minimum	400	<b>0.5</b>
Irish Sea	Gowen & Bloomfield (1996) minimum	560	<b>0.7</b>
Church Island	Hermansen <i>et al.</i> (2001) maximum	1847	<b>2.31</b>
Port Erin	Sanderson <i>et al.</i> (1996) minimum	2398	<b>3.0</b>
Irish Sea	Gowen & Bloomfield (1996) typical	3597	<b>4.5</b>
Danish fjords	Sand-Jensen <i>et al.</i> (1994) typical	3997	<b>5.0</b>
Port Erin	Sanderson <i>et al.</i> (1996) typical	6395	<b>8.0</b>
Menai Strait	Blight <i>et al.</i> (1995) maximum	12 390	<b>15.5</b>
Malin Shelf	Gowen <i>et al.</i> (1998) maximum	12 390	<b>15.5</b>
Port Erin	Sanderson <i>et al.</i> (1996) maximum	12 790	<b>16.0</b>
Irish Sea	Gowen & Bloomfield (1996) maximum	12 790	<b>16.0</b>
Laboratory	This study (Food concentration-A)	<b>100</b>	<i>0.125</i>
Laboratory	Riisgård & Goldson (1997)	<b>810</b>	<i>1.013</i>
Laboratory	Hermansen <i>et al.</i> (2001)	<b>1000</b>	<i>1.251</i>
Laboratory	This study (Food concentration-B)	<b>1000</b>	<i>1.251</i>
Laboratory	Hermansen <i>et al.</i> (2001)	<b>1500</b>	<i>1.877</i>
Laboratory	Riisgård & Goldson (1997)	<b>2246</b>	<i>2.810</i>
Laboratory	Hermansen <i>et al.</i> (2001)	<b>2500</b>	<i>3.128</i>
Laboratory	Hermansen <i>et al.</i> (2001)	<b>3300</b>	<i>4.128</i>
Laboratory	Hermansen <i>et al.</i> (2001)	<b>4100</b>	<i>5.129</i>
Laboratory	Riisgård & Goldson (1997)	<b>4620</b>	<i>5.780</i>
Laboratory	Hermansen <i>et al.</i> (2001)	<b>5000</b>	<i>6.255</i>
Laboratory	Riisgård & Goldson (1997)	<b>8830</b>	<i>11.05</i>
Laboratory	This study (Food concentration-C)	<b>10 000</b>	<i>12.51</i>
Laboratory	Hunter & Hughes (1994)	<b>10 000</b>	<i>12.51</i>
Laboratory	Riisgård & Goldson (1997)	<b>18 500</b>	<i>23.14</i>
Laboratory	Hunter & Hughes (1994)	<b>50 000</b>	<i>62.55</i>
Laboratory	This study (Food concentration-D)	<b>100 000</b>	<i>125.10</i>
Laboratory	Hunter & Hughes (1994)	<b>100 000</b>	<i>125.10</i>

**Analysis of Data Set One.** – Variation of average zoecium area among colonies grown at various food concentrations (Table 1) was assessed visually for scatter plots of mean zoecium area versus food concentrations and visually by comparison with the distribution of equivalent nutritional levels from natural settings (Table 2).

## Data Set Two: zoecial character measurements

**Specimens.** – Clonal replicates of *Electra pilosa* were grown by M.M. Bayer at the Gatty Marine Laboratory in 1992 for a study of polypide regeneration (Bayer *et al.* 1994) and a subset of those specimens was re-measured here. The original source colonies of *Electra pilosa* that were collected growing on fronds of the fucoid macroalga *Fucus serratus* (L.) were collected by Bayer from the lower intertidal of Clachan Seil, Argyll, Scotland (56°18'N05°35'W) and St Andrews Bay, Fife, Scotland (56°21'N02°45'W). The original colonies (genets) were allowed to grow from their algal substratum onto glass slides for ten weeks. Clonal replicates (ramets) were obtained for three different genotypes (genotypes 1, 5 and 7 of Bayer *et al.* 1994) by further growth of the colonies off the glass slides and onto pre-scored cover slips. The pre-scored cover slips were split, providing attached colony fragments that were genetically

identical. The ramets healed, recommenced growth and were allowed to then grow onto experimental glass plates in a tank of filtered seawater. The experiment commenced by removing the attached cover slip and scraping the glass plate to reduce each colony to a section of just 12 zooecia in a three-column  $\times$  four-row zooid array. A replicate of each of the three genotypes was placed into eight culture tanks (two replicates  $\times$  four food concentrations) within a common thermostatically controlled water bath (Fig. 1). The food concentration was varied among the tanks, but all other environmental factors were held constant. Four different food concentrations of the cryptophyte *Rhodomonas* sp. were provided to replicate pairs of tanks (Tank-i versus Tank-ii): Food concentrations were A = 100, B = 1000, C = 10 000 and D = 100 000 cells/mL. After 48 days of growth, with daily replenishment of food concentrations, the colonies were removed, cleaned using freshwater and air-dried prior to storage and subsequent measurement (Bayer *et al.* 1994).



*Fig. 1.* Experimental design of Bayer *et al.* (1994). Replicate tanks in common water bath used to grow bryozoans of three different genotypes (G-1, G-5, G-7), under four different food concentrations (A, B, C, D) for each. Replicate tanks were not systematically arranged, but were allocated randomly to positions within the common water bath.

The following notation is used throughout this paper: G = Genotype (1, 5, 7); F = Food concentration (A, B, C, D); T = Tank (i, ii);  $G \times F$  = Genotype  $\times$  Food concentration interaction;  $\text{Tank}(G \times F)$  = Tank nested within Genotype  $\times$  Food concentration interaction (Fig. 1).

**Data Acquisition** - ANOVA included five balanced observations per colony (tank  $\times$  genotype), for five characters across Food concentrations-B, -C, and -D. Digital images of the zooecia that grew during the 48-day experimental period were captured by video camera and a WILD M8 stereomicroscope (Leica Microsystems, Wetzlar, Germany) at  $\times 50$ . Lower magnification images

( $\times 6$ ) provided maps of each colony (Fig. 2A), and from which, a patch (3 columns [A-C]  $\times$  5 rows [1–5]) of zooecia was selected (Fig. 2A) such that fully mature individuals close to the growing edge could be measured.

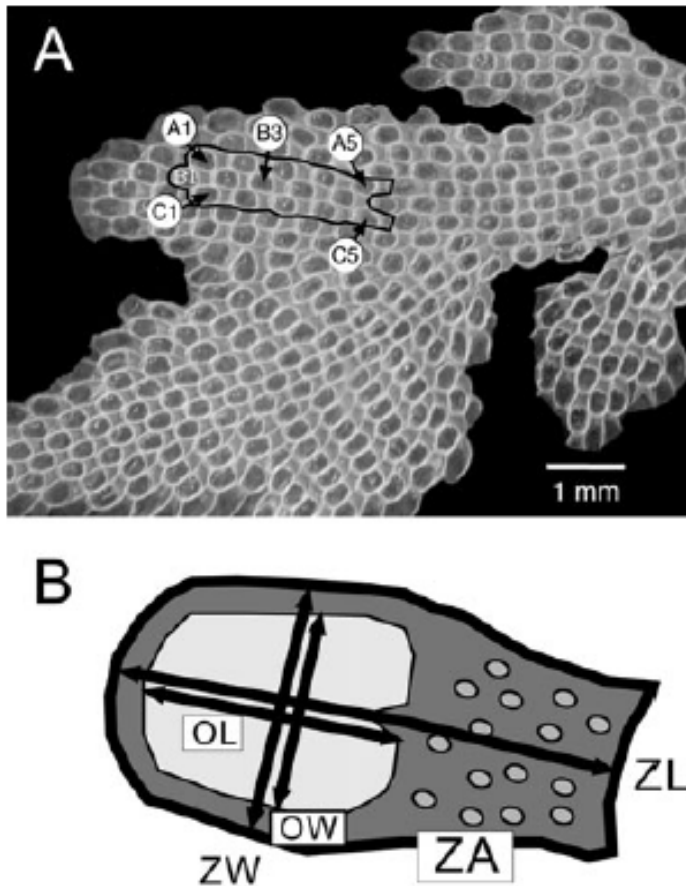


Fig. 2. Specimen of *Electra pilosa* from Bayer *et al.* (1994), with positions of measurements used for Data Set Two in this study. A, colony map of *Electra pilosa* (Genotype-7, Food concentration-D, Tank-i). A patch of fifteen zooecia was chosen (three columns [A-C] by five rows [1–5]) from each colony map (two per colony). Five zooecia were chosen from the patch to be measured in NIH Image. B, diagram of five measurements made for zooecia using NIH Image: Opesia width (OW), Zooecium width (ZW), Opesia length (OL), Zooecium length (ZL) and Zooecium area (ZA).

Within the patch of interest, five zooecia were selected for measurement. These zooecia were not adjacent to one another, and areas of lateral budding and bifurcation of lineal columns were avoided to minimize the possible influence of positional effects (Hageman *et al.* 2002). Digital images ( $\times 50$ ) for each of the five zooecia were saved and five simple morphometric characters were measured (NIH Image version 1.61 software) for each zooecium: Opesia width (OW) and

length (OL), Zooecium width (ZW) and length (ZL), and Zooecium area (ZA) (Fig. 2B). Identical measurements are routinely obtained from well-preserved fossil bryozoans, providing for direct comparison between recent and fossil material. Data are available from the senior author.

**Missing data.** – Specimens maintained at the lowest food concentration (A) were not measured for Data Set Two due to insufficient colony growth. Only two zooecia were measured for the combination of Genotype-7, Food concentration-B, Tank-i, again due to insufficient growth. Without including estimates for three missing data points, the statistical analysis would have violated the assumptions of a balanced anova (Sokal & Rohlf 1995, p. 357). Three values were estimated using Normal Order Deviates. The goal was to use existing data to predict the values of the few missing data points based on assumptions from idealized normal distributions. The intent was to reconstruct a population with an expected variance and mean, not to predict the precise values of unobserved individuals. The calculated missing observations produce a population with a variance and mean that are comparable to other, very closely related populations (with the assumption that the variance of the population in question does not differ significantly from that with which it is being compared).

The procedure developed for estimating missing values for the character *Opesia* width is summarized here for Genotype-7, Tank-i: (1) Record the available values (7BiA1 = 0.268 and 7BiB1 = 0.277), and calculate their average (0.273). (2) Estimate a standard deviation for the population with values from very closely related populations. Standard deviation for Food concentration-B: Genotype-1 × Tank-i = 0.0119, Genotype-1 × Tank-ii = 0.0196, Genotype-5 × Tank-i = 0.0177, Genotype-5 × Tank-ii = 0.0251, Genotype-7 × Tank-ii = 0.0069). (3) Calculate the *average* standard deviation for all of these from Step 2 (0.0162) and use as the target (final) standard deviation of group with missing data. (4) Look up the Normal Order Deviate for a sample of five observations (−1.163, −0.495, 0.0, +0.495, +1.163) (Rohlf & Sokal 1981, table 37). (5) Estimate the maximum value in the group with the estimated standard deviation (0.0162) multiplied by the largest normal order deviate (1.163) and then the result (0.0188) is added to the average of the known values:  $0.273 + (0.0162 * 1.163) = 0.291 = 7Bi$  Est. Max. (6) Estimate the minimum value in the group by subtracting the equivalent value:  $0.273 - (0.0162 * 1.163) = 0.254 = 7Bi$  Est. Min. (7) Estimate a third value using the two known values and the two values estimated in Steps 6 and 7, plus a 'starter value' for the third unknown designated as 7BiVar. Use the mean of the two known values [0.273] as a starting value. (8) A new standard deviation = 0.0141 results for this population using the starter value 0.273. This value is less than the expected standard deviation of 0.0162 from Step 3, so a new starter value (0.240) was used in the next estimate. (9). A new standard deviation = 0.020 results for this population using the starter value 0.240. This value is greater than the expected standard deviation of 0.0162 from Step 3. (10) This process is repeated, iteratively, with estimated values until the standard deviation from the estimated population approximates the target standard deviation of 0.0162, which for this study was 7BiVar = 0.253. (11) The values derived for missing values of Genotype-7, Food concentration-B and Tank-i, were entered into the revised data matrix as: 7BiMax = 0.291, 7BiMin = 0.254 and 7BiVar = 0.253.

**Analysis of Data Set Two.** – The five morphometric zooecial characters were analysed in three ways:

1. Excluding Food concentration-A (insufficient growth to measure), the log-transformed data for each zooecium were projected into a single, new coordinate system using principal



2. Each character was evaluated for significance among group means using a mixed model, two-way, nested anova. The factors considered were Genotype (fixed), Food concentration (fixed), with the factor of Tank (random) nested within the Genotype  $\times$  Food concentration interaction (Table 3). Mean squares for each factor and interaction were obtained with SuperAnova version 1.11, and appropriate F-value calculations were completed in a spreadsheet using equations in Table 4 from Zar (1999, appendix A.4). Probabilities were obtained using SISA – Simple Interactive Statistical Analysis (Uitenbroek 1997). The percentage of variance represented by each factor and their interactions also was calculated using the mean sum of squares for each character (Sokal & Rohlf 1995, pp. 212–214; Hageman *et al.* 1999).

Table 3. Example of ANOVA table (for Opesia width), including values for degrees of freedom (df), sum of squares (SoS), mean sum of squares (MS), F-value (calculations in Table 4), probability (P-value), number of observations (n), variance (Var) and the percentage of the total variance accounted for by each level (%Var).

[illegible]

Table 4. Model for the calculation of F-values and degrees of freedom ( $\nu 1$  and  $\nu 2$ ) for a Mixed Model Analysis of Variance with three factors; two primary factors that are fixed (Genotype and Food concentration) and one random factor (Tank) nested within the interaction of the other two (after Zar 1999: appendix A.4, p. 476).

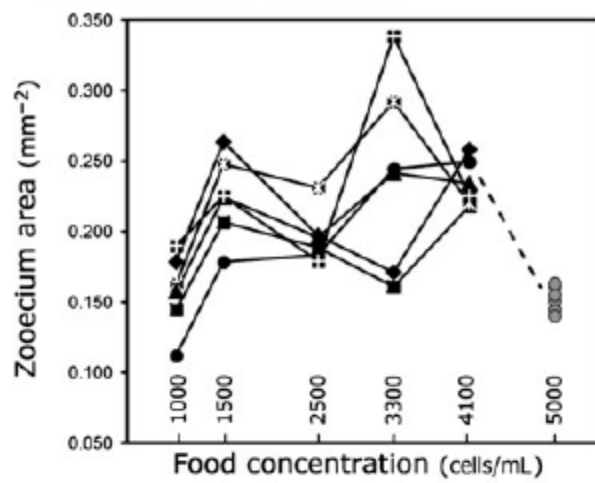
Factors	F-value	$\nu 1$	$\nu 2$
Genotype <sub>(fixed)</sub>	$MS_G / MS_{\text{Tank}(G \times F)}$	$df_G$	$df_{\text{Tank}(G \times F)}$
Food concentration <sub>(fixed)</sub>	$MS_F / MS_{\text{Tank}(G \times F)}$	$df_F$	$df_{\text{Tank}(G \times F)}$
G $\times$ F interaction	$MS_{(G \times F)} / MS_{\text{Tank}(G \times F)}$	$df_{(G \times F)}$	$df_{\text{Tank}(G \times F)}$
Tank <sub>(random)</sub> (G $\times$ F)	$MS_{\text{Tank}(G \times F)} / MS_{\text{residual}}$	$df_{\text{Tank}(G \times F)}$	$df_{\text{residual}}$

## Results, Data Set One: zooecium area

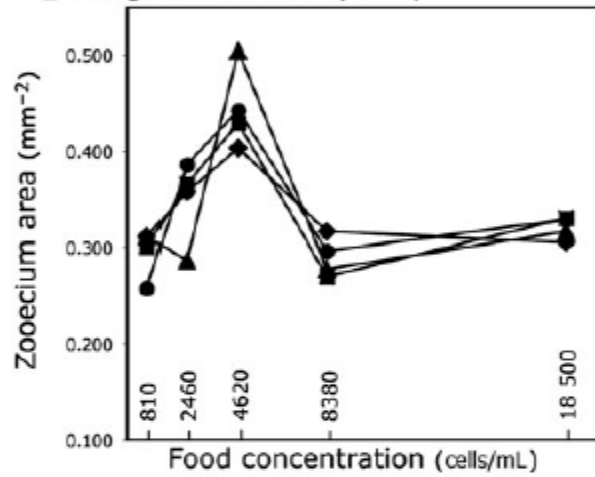
### Zooecium area versus food concentration

Based on original differences in experimental designs, values for Food concentration are plotted on three different scales in Figure 3: 0–5500 cells/mL (Fig. 3A, Hermansen *et al.* 2001), 0–20 000 cells/mL (Fig. 3B, Riisgård & Goldson 1997) and partially logarithmic 0–100 000 cells/mL (Fig. 3C, Bayer *et al.* 1994). Yet, all studies yield the same general pattern in zooecium size and variation (Table 1): (1) smallest zooecium at the lowest food concentration; (2) maximum zooecium size and variability at food concentrations between 3000–10 000 cells/mL; and (3) less variable, submaximum-sized zooecia at all concentrations above 10 000 cells/mL.

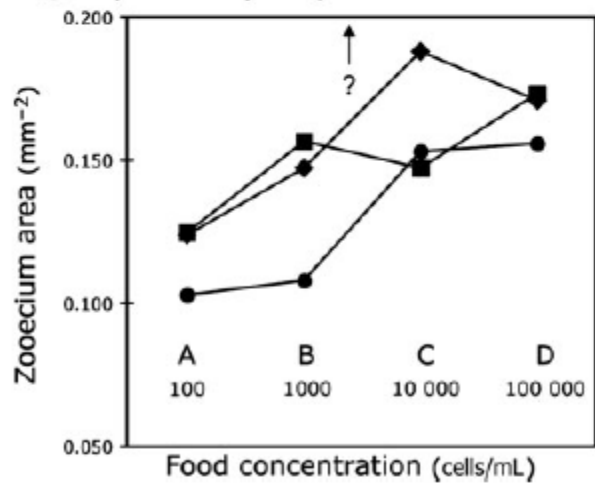
**A** Hermansen *et al.* (2001)



**B** Riisgård & Goldson (1997)



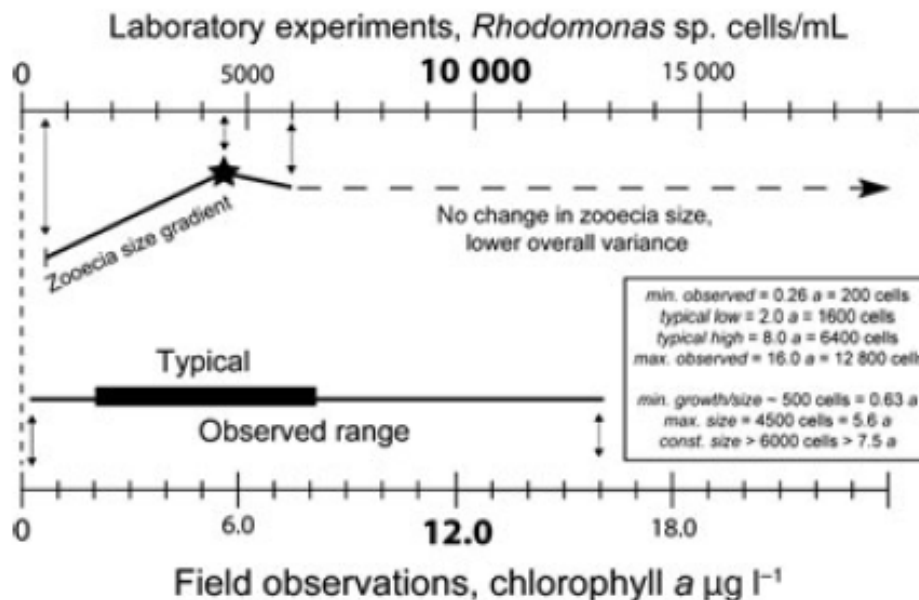
**C** Bayer *et al.* (1994)



**Figure 3.** Scatter plots of Food concentration (cells of *Rhodomonas* sp.) versus Average zooecium area (per colony) from three independent studies. Lines connect colonies of the same genotype (clone) grown under different food conditions (except 3A between 4100 and 5000, where dashed line connects group means). Horizontal scales vary among graphs based on original experimental design.

## Comparison with the natural environment

Food concentrations (number of cells/mL) used in these laboratory studies are comparable to equivalent nutrition levels (chlorophyll *a*) observed in nature from relevant settings (Table 2, Fig. 4). Values of chlorophyll *a* observed in the wild in the Irish Sea range from 0.26–16.0  $\mu\text{g/L}$  (Blight *et al.* 1995; Gowen & Bloomfield 1996; Gowen *et al.* 1998; Hermansen *et al.* 2001), which corresponds to 200–12 800 cells/mL for *Rhodomonas* (Table 2, Fig. 4). More typical values observed in shallow waters of the Irish and North Seas were 2.0–8.0  $\mu\text{g/L}$ , corresponding to 1600–6400 cells/mL for *Rhodomonas* (Sand-Jensen *et al.* 1994; Gowen & Bloomfield 1996; Sanderson *et al.* 1996; Hermansen *et al.* 2001).



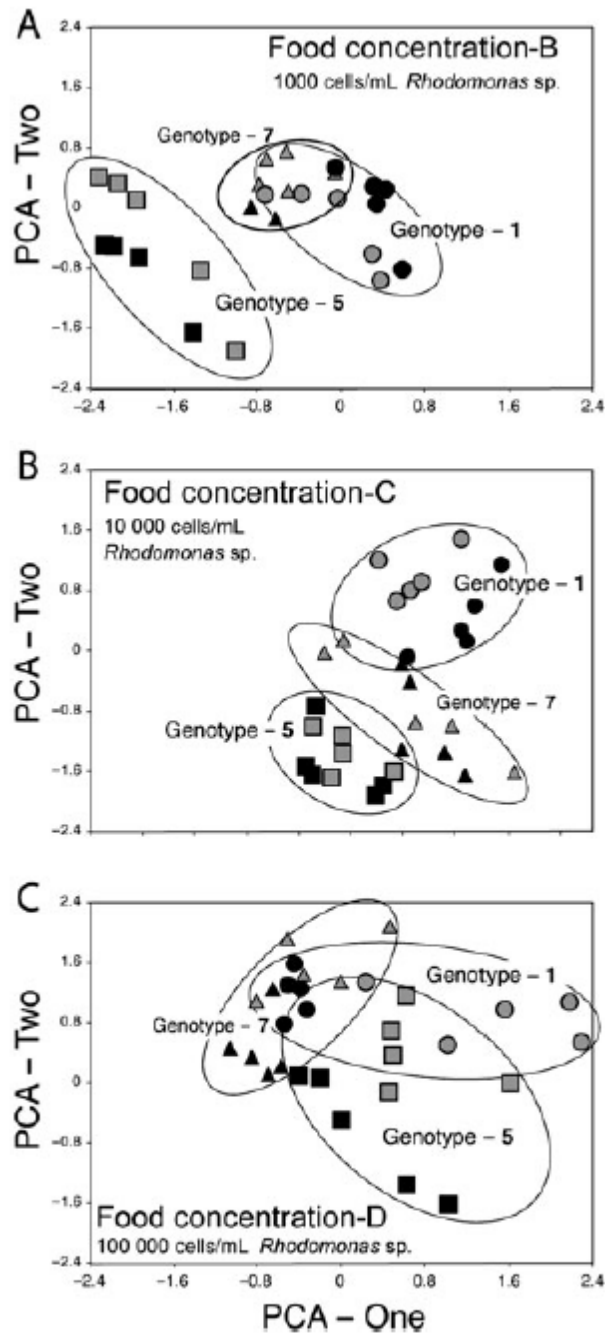
**Figure 4.** Generalized representation of results from Figure 3 for Food concentration versus Zooecium size, and comparison to nutrient levels observed in the natural environment (Table 2).

These natural levels encompass the critical food concentrations associated with zooecium size change observed in laboratory specimens (Fig. 4), i.e. the lower threshold of growth (500 cells of *Rhodomonas*/mL = 0.63  $\mu\text{g/L}$  chl *a*), the gradient of zooecium size change (500–4500 cells/mL = 0.63–5.6  $\mu\text{g/L}$ ), and the sustained submaximum zooecia size above (> 6000 cells/mL = 7.5  $\mu\text{g/L}$ ).

## Results, Data Set Two: morphometric characters

### Principal component analysis

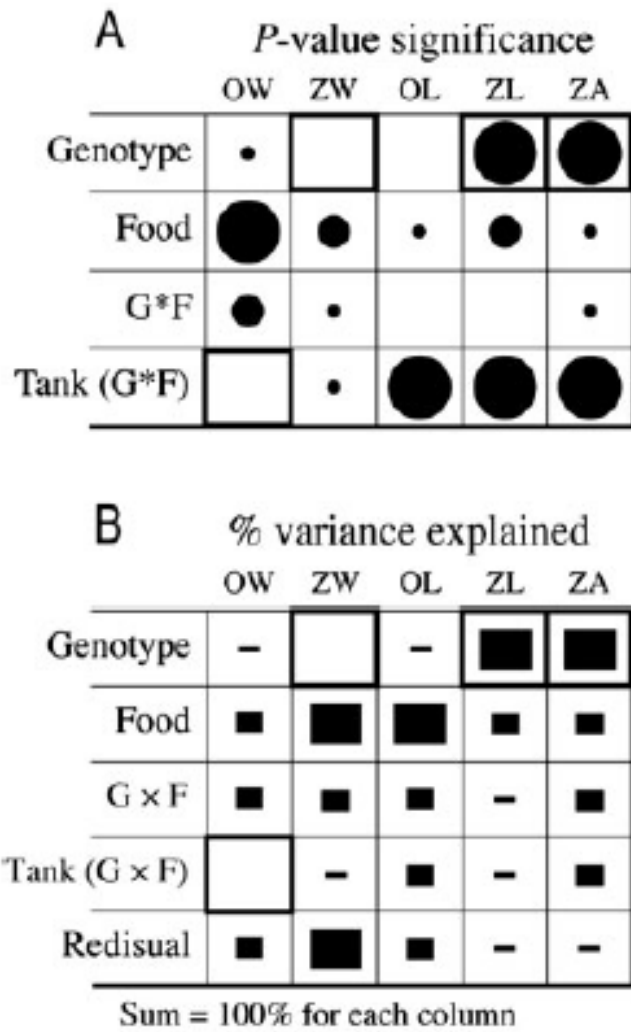
Results of the PCA from specimens of Bayer *et al.* (1994) are shown in Figure 5. An increase in zooecium size between Food concentrations-B (1000 cells/mL) and -C (10 000 cells/mL) is evident in the comparison of Figure 5A, B by the shift to the right (larger size) with increased Food concentration. PCA shows a clear separation of both of the main effects for Genotype and Tank at Food concentration-C (Fig. 5B). The first two PCA axes represent 89.9% of the variation and the first PCA axis represents all five characters weighted approximately equally (coefficients ZA = 0.35, OL = 0.30, ZL = 0.28, ZW = 0.22, OW = 0.20). The second PCA axis is a contrast between width versus length (OW = -0.45, ZW = -0.43 versus ZL = 0.33, OL = 0.15, ZA = 0.13). PCA axis three (not plotted here) accounted for 8.5% of the total variance and represents a contrast between Opesia length and Zooecium area and length (OL = -1.30 versus ZA = 0.65, ZL = 0.49).



**Figure 5.** Principal component analysis calculated using Data Set Two and five morphological variables (first two axes account for 89.5% of the total variance). Data for Food concentrations are plotted separately. A, food concentration-B; B, food concentration-C; C, food concentration-D. There is a shift from smaller zooecia to large zooecia on (x-axis) from Food concentrations-B to -C. Food concentration-C shows a separation of Genotype and also a slight separation of Tanks. Ellipses highlight groups, but do not have statistical significance.

## Two-way nested anova

The mixed model nested anova (Fig. 6) can be summarized as follows. The primary factor Genotype was most significant for the characters Zooecium length and area (ZL, ZA) (Fig. 6A). Genotype also accounted for the greatest amount of variance for Zooecium length and area (ZL, ZA) (Fig. 6B), but was least important for the character Zooecium width (ZW) (Fig. 6B). Food concentration was significant (Fig. 6A) and accounted for a large proportion of the variation for all characters measured (Fig. 6B). Overall, Genotype  $\times$  Food concentration interaction displayed a lesser degree of significance than the main effects (Fig. 6A). However, it is notable that the Genotype  $\times$  Food concentration interaction did account for a moderate proportion of the variances for each of the separate characters (Fig. 6B). The primary factor Tank, which was randomly distributed within each Genotype  $\times$  Food concentration group, was highly significant for characters associated with length, ZL and ZA, and Opesia length (OL) (Fig. 6A) and accounted for a low to moderate amount of variance for all characters except Opesia width (OW) (Fig. 6B). The residual variance attributable to other effects, which are not accounted for in the model, occurred at a moderate level for all characters (Fig. 6B), but was greatest for Zooecium width (ZW). Individual characters Zooecium length and area (ZL and ZA) for Genotype were the only combinations (Fig. 6A) within the analysis that accounted for both a very high level of significance and the greatest proportion (Fig. 6B) of variance. Two combinations displayed minimal levels of importance (Zooecium width, ZW for Genotype and Opesia width, OW for Tank) (Fig. 6). Among the primary factors, Tank had the most characters (OL, ZL and ZA, Fig. 6A) that were highly significant, but Food concentration was significant across all characters (Fig. 6A), and consistently explained a high proportion of variance across all characters (Fig. 6B).



**Figure 6.** Results for anova for Data Set Two. A, summary of significance levels for five morphometric characters for each source of variation. Values of  $P < 0.0001$  (large circles) are highly significant; values of  $0.001 < P < 0.0001$  (medium circle) are significant; values of  $0.05 < P < 0.001$  (small circles) are marginally significant; and values of  $P > 0.05$  (blank) are not significant. B, summary of the percentage of total variance accounted for by each factor for a given character (columns sum to 100%). Large rectangles indicate  $> 30\%$  of the variance is accounted for by the factor/effect, mid-sized rectangles between 10 and 30%, a dash indicates 5 to 10% of the total variance is accounted for by the factor/effect, and a blank represents less than 5%. Three highlighted cells (OW-Tank, ZW-Genotype, and ZL-Genotype) displayed a strong congruence with results for the  $P$ -values (cf. Fig. 6A).



## Magnitude of change

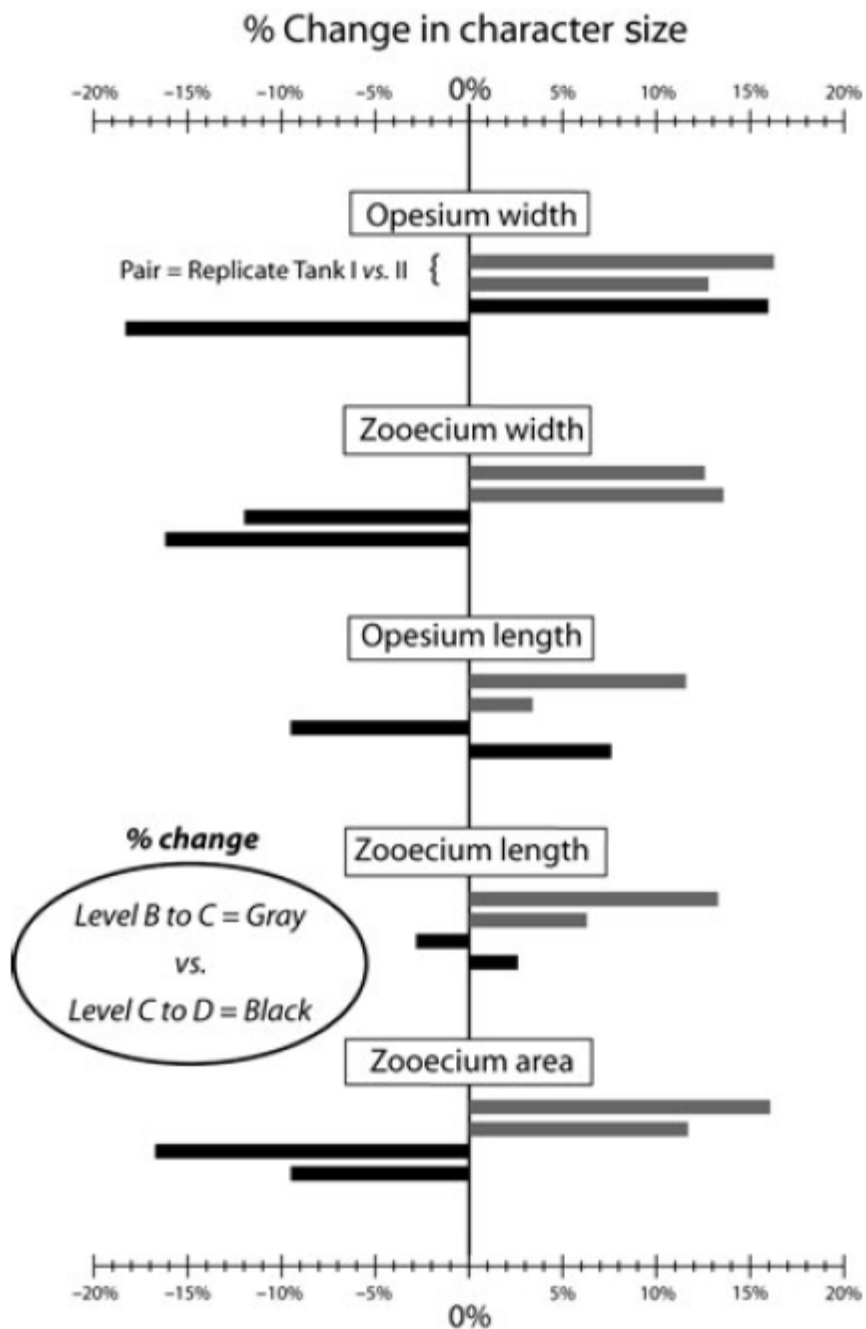
The magnitude of change (percentage size difference) between results from Food concentration treatments (Tables 5, 6) is summarized for each character in Figure 7. Averages for all five characters showed increases in size between Food concentrations-B and -C (Fig. 7, grey bars to right). The response between Food concentrations-C and -D was mixed, but included many negative changes in size (Fig. 7, black bars to the left). This trend was also evident in a tally of how clonal replicates of the same genotype responded to Food concentration treatments in replicate Tanks (Fig. 8). Most of the individual colonies increased in size for all characters between Food concentrations-B and -C (Fig. 8, first column). The response to the increase of Food concentrations between -C and -D was less consistent (Fig. 8).

*Table 5. Mean values (mm) for five characters (OW = Opesia width, ZW = Zooecium width, OL = Opesia length, ZL = Zooecium length, ZA = Zooecium area) at three Food concentrations (B = 1000, C = 10 000 and D = 100 000 cells/mL); n = 30 observations for each Food concentration.*

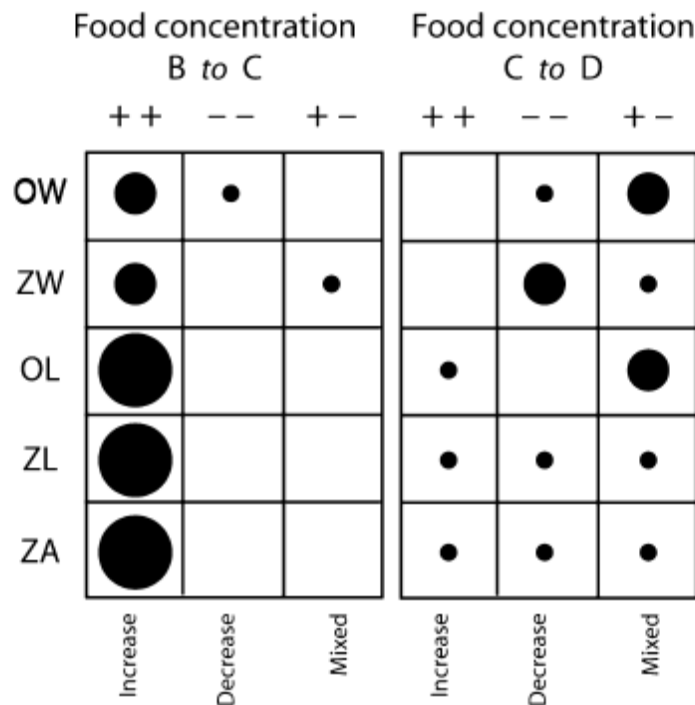
Food concentration	OW	ZW	OL	ZL	Area
B	0.274	0.307	0.347	0.536	0.141
C	0.299	0.332	0.395	0.597	0.162
D	0.271	0.306	0.402	0.614	0.158

**Table 6.** Summary of the percentage of change in character size from one Food concentration to another used in Figure 7. Data are pooled for Genotype and Tank for each Food concentration (B = 1000, C = 10 000 and D = 100 000 cells/mL). Averages are geometric means from absolute values.

Change between food concentrations	OW	ZW	OL	ZL	Area	Average for all characters
B to C	109.1%	108.3%	113.8%	111.5%	114.8%	111.5%
	+9.1%	+8.3%	+13.8%	+11.5%	+14.8%	<b>+11.5%</b>
C to D	90.4%	92.0%	101.8%	102.8%	97.3%	96.8%
	-9.6%	-8.0%	+1.8%	+2.8%	-2.7%	<b>-3.2%</b>



**Figure 7.** Effect of changes in Food concentration on each morphological character. Grey bars reflect the percentage size change associated with change from B = 1000 to C = 10 000 *Rhodomonas* cells/mL. Black bars reflect the percentage size change associated with change from C = 10 000 to D = 100 000 cells/mL.



**Figure 8.** Response for each character in Data Set Two to an increase in Food concentration. Two positive symbols ‘++’ indicate conditions, where both clonal replicates increased in size with treatment. Two negative symbols ‘—’ indicate conditions where both clonal replicates decreased in size with treatment. The size of dot represents the number of genotypes represented by each: large = 3, medium = 2, small = 1 and empty = 0.

Responses of zoecium size change to Food concentration treatments (Table 6) demonstrate a range of 8.3–14.8% increase in size morphological characters associated with a change in Food concentration from B = 1000 to C = 10 000 cells/mL (Table 5), with an overall average of an 11.5% increase in size. In contrast, a change in Food concentration from C = 10 000 to D = 100 000 cells/mL (Table 6) produced a range of a 9.6% decrease to a 2.8% increase in size. The average for all characters is a 3.2% decrease in size (Table 6).

## DISCUSSION

### Summary results

The overall patterns of response observed in this study are perhaps more important to palaeobiological interpretations than are absolute values. With the experimental designs employed in this and related studies (very closely related specimens with simple morphologies, grown under controlled environmental conditions), one would not expect robust patterns of

morphological variation. Therefore, we consider even subtle patterns in the distribution of morphological variation noteworthy and arguably relevant in a palaeobiological context.

**Colony growth rate.** – Many previous studies of bryozoans have documented an increase in growth rate (number of new zooecia per interval of time) with increased availability of food (e.g. Jebram 1980; Hunter & Hughes 1994; Bayer *et al.* 1994; Riisgård and Goldson 1997; Hermansen *et al.* 2001). In the absence of competition, this relationship appears to continue with increased food availability to an as yet unidentified level. A lower limit for colony growth is near or below 500 cells/mL *Rhodomonas* = 0.63 µg/L chl *a*, which is itself near the lower limit of nutrition concentrations observed in the natural environment of *Electra pilosa* (Table 2).

**Size of zooecia.** – Food concentration yields a significant and predictable effect on the phenotype of *Electra pilosa*. Responses of Zooecium size to Food concentration can be summarized in four conditions. Threshold values for Food concentrations can be generalized from the observed patterns in zooecium size variation (Table 2, Fig. 4): (1) minimum size: 500 cells/mL = 0.63 µg/L chl *a*; (2) positive size gradient: 500–4500 cells/mL = 0.63–5.6 µg/L chl *a*; (3) maximum size: 4500 cells/mL = 5.6 µg/L chl *a*; and (4) more constant, but less than maximum size: > 6000 cells/mL = 7.5 µg/L chl *a*. All of these conditions occur well within natural levels of food concentration (Table 2, Fig. 4).

Very low food concentrations were insufficient for the bryozoans to flourish, resulting in stunted colonies with small zooecium sizes. At low to intermediate food concentrations, there was a direct effect on zooecium sizes (Table 1, Figs 3,4). Although important to confirm, these results are not inherently surprising.

Maximum zooecium sizes occurred in *Electra pilosa* at submaximum food concentration, whereas submaximum zooecia sizes occurred at higher food concentrations (Table 1, Figs 3,4). This suggests that up to a threshold food concentration, a priority is placed on developing larger individuals (polypides) in a colony. Above the threshold level, priority is then placed on the addition of new individuals of an optimal but submaximum size. This threshold may be associated with the trade off of potential for food sequestration per unit size versus the cost of growth per unit within a colony. However, existing data and our present understanding of energy transfer/costs within colonies are not yet sufficient to model or test this conjecture.

Minor but systematic variation in zooecium size among Tanks at the same Food concentration (Fig. 5) in the specimens of Bayer *et al.* (1994) is likely a reflection of a consistent but small-scale variation of conditions within the water bath. Such positional effects of Tanks within the water bath and placement/arrangement of specimens within each Tank are to be expected despite attempts to minimize these with water bath stirrers and randomization of positions. These results are not surprising given the sensitivity of zooecium size at low- to mid-levels of Food concentration. Replicate Tanks allow this source of variation to be isolated analytically and demonstrate that they account for a significant, but only very small, percentage of the total variance (Fig. 6).

Previous studies that have shown no relationship between zooecium size and food concentration (nutrition levels) in *Electra pilosa*, *Conopeum seurati* and *Celleporella hyalina* (Jebram 1973, 1975, 1980; Jebram & Rummert 1978; Okamura 1987; Hunter & Hughes 1994; O'Dea & Okamura 1999, 2000b) either reported food concentrations higher than the threshold observed in this study (> 6000 cells/mL of *Rhodomonas*) or did not report nutrition levels. It is

possible that the applied food concentrations were well above the critical value for zooecia variation.

**Variability of zooecia.** – The magnitude of variation of zooecium size within colonies reflected the trend in zooecium size. Maximum zooecia variance was coincident with maximum average zooecium size at the threshold food concentration (Figs 3, 4), 4500 cells/mL = 7.51 µg/L chl *a*. Minimum zooecia variance is observed under higher food concentrations with sub-maximum zooecium size, greater than 6000 cells/mL = 7.51 µg/L chl *a* (Figs 3,4). The interval of zooecium size change associated with low to threshold food concentration showed intermediate levels of variation. These results are important for studies that rely on analysis of within-colony variation. In the absence of other factors, variation in zooecium size is minimal and unchanging at moderate to high nutrition levels: greater variation in zooecium size is expected at the threshold food concentration and levels below.

**Response of characters.** – The genotype appears to have greater control of characters associated with Zooecium length and Zooecium area (ZL, ZA; Fig. 6). Characters related to the width of zooecia have less correspondence with the genotype, which may itself reflect space constraints during budding (Hageman *et al.* 2002).

## Broader implications

**Recognition of genotypes.** – Small-scale genetic differences among sexually produced colonies (unique genotypes) were detectable in the skeletal phenotype in this study. Similar results have been reported in both modern and fossil Bryozoa (Cheetham *et al.* 1993, 1995; Hunter & Hughes 1994; Hageman *et al.* 2002). Our results provide robust support for the conjecture that a significant portion of morphological variation within species of bryozoans can be unequivocally attributed to genotype, even under circumstances of strong environmental influences on the expression of phenotype.

**Palaeoecological studies.** – Studies of palaeoclimate and palaeoseasonality that assess changes of within-colony zooecium size variation as a proxy for (palaeo)seasonality assume that temperature is the primary control on zooecium size variation (O'Dea & Okamura 2000a,b; O'Dea 2005). The observed linear relationship between zooecium size and temperature can be used in studies of MART (mean annual range of temperature) (O'Dea & Okamura 2000a). Considerable data now document a correlation between zooecium size and temperature (Menon 1972; Hunter & Hughes 1994; Okamura 1987; O'Dea & Okamura 1999, 2000a; O'Dea 2005; Lombardi & Cocito 2006; Amui-Vedel *et al.* 2007; O'Dea *et al.* 2007). However, results from this and other studies of *Electra pilosa* suggest that food concentration can also have an effect on zooecium size and within colony variance at low- to mid-level concentrations. In nature, food level-variation, can be linked directly to seasonal parameters, including, but not limited to, water temperature (Blight *et al.* 1995; Gowen & Bloomfield 1996; Gowen *et al.* 1998). Thus, it may be possible that nutritional fluxes above and below critical concentrations (~7.51 µg/L chl *a* for observed *Electra pilosa*) can enhance seasonality signals observed in MART studies. However, nutritional fluxes across critical food concentrations could also confound interpretations in MART analyses if temperature were not a correlative factor with food concentration.

## Future Work

Several questions are raised by these results:

1. Do trends observed for *Electra pilosa* maintain for other species of bryozoans?
2. How well do trends observed under laboratory conditions reflect responses to natural conditions?
3. What are the inter-relationships between food concentration and other proximate factors such as temperature and physical disturbances?
4. Do other factors besides food, temperature or disturbance affect zooecium size?

These questions can be addressed in two ways, with laboratory-controlled settings such as those described here and in field experiments where relevant physical and biological parameters are closely monitored and documented to establish correspondence with zooecia developed under known conditions (e.g. Okamura 1985; Hermansen *et al.* 2001).

It has been shown that monoculture food sources for laboratory experiments are not optimal for growth (Hart & Santer 1994; Picard & Lair 2000). Food concentration values in this study do not include non-photosynthetic contributors to natural food sources for Bryozoa such as naked flagellates and ciliates and metazoan larvae (Best & Thorpe 1994). Nutrient levels in nature are the result of many inputs that vary over space and time (Best & Thorpe 1994; Gowen & Bloomfield 1996; Hermansen *et al.* 2001). Indeed, zooecia in this study from portions of colonies grown in the wild are larger than those of the same genotype grown in all monoculture laboratory conditions in each of these studies (Bayer *et al.* 1994; Riisgård & Goldson 1997; Hermansen *et al.* 2001). Monocultures are used to simplify experimental designs, but similar studies need to be performed using food types more representative of natural conditions. Potential interactions between factors that affect zooecium size (food concentration, current velocity and temperature) need to be better constrained through critical intervals. Also, experiments need to be performed with various colony sizes. Although no evidence was observed in the data reported here, a 'starter effect' may be present because all laboratory colonies began with 12 zooids. It is unclear what the effect of varied food availability would be during the development of much larger colonies, i.e. the unknown of nutrient use, storage and transfer within colonies.

## Summary

Varying Food concentration within clones of the bryozoan *Electra pilosa* has a non-linear effect on the size of individual zooecium (skeleton) within the colony. A lower threshold exists where minimal growth takes place and the smallest zooecia form (Table 7). A range of increasing food concentrations produces a gradient of increasing zooecia size up to a threshold where maximum zooecia sizes are produced. Maximum size also corresponds to the greatest variance (Table 7). With increased concentration, a plateau in zooecia size is reached that is smaller than the maximum, but less variable.

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**Table 7.** Generalized summary of the effects of varied Food concentration on the growth rate, size and size variance of *Electra pilosa*. Single symbol ‘•’ = minimum influence, five symbols ‘•••••’ = maximum. Observations based on laboratory grown (cells/mL), chlorophyll *a* levels (µg/L) are calculated equivalents.

Food concentration cells	Growth chlorophyll <i>a</i>	Zooecium rate	Zooecia size	Variance
0–100	0–0.125	•	•	••
100–3000	0.125–3.753	••	••	••
3000–6000	3.753–7.506	•••	•••••	•••••
>> 6000	>> 7.506	•••••	•••	•

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Studies that document the effect of environmental parameters on the skeletal morphology of individual zooecia within and among closely related colonies provide insights into the origin of the phenotype. Such data can greatly enhance studies of microevolution and palaeoenvironmental analysis.

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